UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

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MEMORANDUM

SUBJECT: EPA's Response to Comments Received on the April 28, 2016, Notice for a Pesticide Product Application with a New Active Ingredient *Wolbachia pipientis*, ZAP Strain in Male *Aedes albopictus* (Asian Tiger Mosquito) (EPA File Symbol 89668-U) - (Docket ID Number: EPA-HQ-OPP-2016-0205; FRL-9945-49)

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BACKGROUND

On July 26, 2013, the U.S. Environmental Protection Agency (EPA or the Agency) issued experimental use permit 89668-EUP-1 to MosquitoMate, Inc. (MosquitoMate) for the new microbial pesticide *Wolbachia pipientis* ZAP strain (also referred to as *w*Pip strain) for use in the Asian tiger mosquito *Aedes albopictus* (EPA-HQ-OPP-2013-0254-0006; 78 FR 56227). The *Wolbachia* ZAP strain does not naturally occur in wild *Aedes albopictus* populations. When ZAP-infected male mosquitoes mate with ZAP-free wild females, the females produce non-viable eggs, leading to a reduction in the *Aedes albopictus* population upon recurrent release of ZAP-infected males.

In accordance with the conditions of 89668-EUP-1, MosquitoMate released *Wolbachia* ZAP-infected *Aedes albopictus* at test sites in Kentucky, California, and New York. The EUP was subsequently extended and amended in 2014 (EPA-HQ-OPP-2013-0254-0010; 79 FR 49774) to allow for weekly releases of *Wolbachia* ZAP strain-infected male *Aedes albopictus* at the original test sites and six additional sites in California. Testing was permitted for the sites in California, Florida, and Kentucky until October 31, 2015, and until September 30, 2016 for the sites in New York.

89668-EUP-1 allowed MosquitoMate to collect information (e.g., product performance data) necessary to support a pesticide registration application under section 3 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

On February 5, 2016, EPA received an application for registration of a pesticide product containing the new active ingredient *Wolbachia pipientis*, ZAP strain for use in *Aedes albopictus* from MosquitoMate (EPA File Symbol 89668-U). In accordance with 40 CFR § 152.102 and pursuant to FIFRA § 3(c)(4), EPA published a Notice of Receipt (NOR) for the registration application in the *Federal Register* of April 28, 2016 (81 FR 25401). In response to this publication, EPA received 10 public comments that consist of a mix of negative, neutral, and positive comments from private citizens, a company (Oxitec, Ltd.), and a non-governmental organization (Center for Food Safety). EPA appreciates all of the comments received, and its primary focus below is to reiterate and respond to the negative and neutral comments.

PUBLIC COMMENTS AND EPA'S RESPONSE

Because some of the comments highlighted similar issues, EPA grouped these comments and generated one response to each grouping. When grouping text from multiple sources, EPA provides specific details as to where the text originates. A substantial number of comments were previously addressed by EPA in the context of responding to comments received for the amendment and extension of an experimental use permit for a different strain of *Wolbachia* and its use in *Aedes aegypti* (EPA-HQ-OPP-2015-0374-0020; U.S. EPA, 2016). EPA will direct readers to the responses provided in its response to comments on the experimental use permit whenever appropriate.

I. PRODUCT DESCRIPTION

Comments

From EPA-HQ-OPP-2016-0205-0002 (Anonymous) - … [D]ispursing [sic] Genetically modified mosquitos has never been proven safe or effective.

From EPA-HQ-OPP-2016-0205-0005 (Anonymous) - I'm 100% against this plan to release GMO mosquitos in FL. […]

EPA's response

The subject of this registration application is the ZAP strain (*w*Pip) of the bacterium *Wolbachia pipientis*. This particular strain has been used to infect the Asian tiger mosquito *Aedes albopictus*. Neither the bacterium nor the mosquito have been genetically engineered in the process. Below, EPA provides a short description of the approach employed by the subject of the current registration application. Discussion of other approaches to mosquito control can be found in the August 30, 2016 response to comments regarding experimental use permit 88877-EUP-2 (EPA-HQ-OPP-2015-0374-0020).

Subject of current registration application
Wolbachia pipientis-infected mosquitoes intended to suppress populations of *Aedes albopictus* mosquitoes (registration application 89668-U).

Purpose:	Population suppression
Mechanism:	Mosquito eggs do not hatch due to cytoplasmic incompatibility
Mosquito species:	*Aedes albopictus*
Bacterium:	*Wolbachia pipientis,* ZAP (*w*Pip) strain
Released individuals:	Males, non-biting

The *Wolbachia pipientis* ZAP-infected mosquitoes are intended to suppress the populations of *Aedes albopictus*. The *Wolbachia* bacterium is naturally occurring in the mosquito species *Culex pipiens* from which it was extracted and subsequently microinjected into embryos of *Aedes albopictus*. These are then reared as a colony in isolated containment facilities that follow Arthropod Containment Level-1 procedures (Tabachnick, 2006). The presence of the ZAP strain in these mosquitoes is confirmed by detecting their genetic fingerprint, using polymerase chain reaction (PCR). Non-biting *Aedes albopictus* males are then sorted from the lab colony by size differentiation and visual inspection, and shipped to the release sites where they can mate with wild-type females that do not carry this particular strain of *Wolbachia*. Through cytoplasmic incompatibility, the embryos from these matings die and the eggs do not hatch (Weeks, 2015). Neither the bacteria nor the mosquitoes are genetically engineered in the process. The *Wolbachia pipientis*, ZAP strain in these mosquitoes is regulated as a microbial pesticide by EPA under

FIFRA (U.S. EPA, 2013). EPA issued, amended, and/or extended other experimental use permits using this same technology in *Aedes aegypti* [1] and *Aedes polynesiensis* in 2012. [2]

II. REGULATORY OVERSIGHT

Comments

From EPA-HQ-OPP-2016-0205-0011 (Oxitec, Ltd.) - FIFRA requires that EPA can only register a pesticide if, *inter alia*, it determines that the pesticide (1) will perform its intended function without unreasonable adverse effects on the environment, and (2) when used in accordance with widespread and commonly recognized practice, will not generally cause unreasonable adverse effects on the environment. (7 U.S.C. § 136a(c)(5)) FIFRA defines "unreasonable adverse effects on the environment," in pertinent part, as "any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide". (7 U.S.C. § 136(bb)) As is discussed in detail below, granting a full FIFRA Section 3 registration for a pesticide product consisting of *Wolbachia pipientis*, ZAP strain present at 100% in male *Aedes albopictus*, with uncontained release to the environment of unlimited numbers of modified mosquitoes carrying *Wolbachia pipientis* may entail significant undefined risk to human health and the environment. EPA is obligated, pursuant to FIFRA, to have a rational basis for any determination that the risks entailed with the intended function and use of this pesticide, if registered, will not cause unreasonable adverse effects on humans or the environment. Thus, to meet its obligations under FIFRA, prior to granting a registration for the new pesticide active ingredient *Wolbachia pipientis*, ZAP strain, which is proposed to be used at 100% in male *Aedes albopictus*, EPA must conduct a sufficiently rigorous assessment of the potential adverse impacts to human health and the environment that the uncontained release of these mosquitoes may entail. Oxitec herein discusses some of the serious and significant human health and environmental risks that must be addressed prior to the granting of a registration for *Wolbachia pipientis Aedes albopictus* mosquitoes.

At the outset, we note that Oxitec has developed a different genetic insect control technology that has been demonstrated to be efficacious in significantly reducing the population of disease-carrying mosquitoes (>90% in the Cayman Islands, Brazil, and Panama). Because Oxitec's insect control technology utilises genetic engineering of the insect genome, it has been determined that it is to be regulated by the U.S. Food and Drug Administration as a new animal drug. It is the oft-stated policy of the U.S. government that it regulates the products of biotechnology on the basis of a "risk-based, scientifically sound approach . . . that focuses on the characteristics of the biotechnology product and the environment into which it is being introduced, not the process by which the product is created." Exercise of Federal Oversight Within Scope of Statutory Authority; Planned Introductions of Biotechnology Products Into the Environment, 57 Fed. Reg. 6753 (Feb. 27, 1992) (this seminal statement is repeated throughout the policy statement, see, e.g., id. at 6754-55, 6755, 6756, 6757, and 6760). Notwithstanding this consistently stated position, the reality is that Oxitec's self-limiting mosquitoes have been subjected to a mandatory pre-market approval regulatory process at FDA that has been much more onerous than the

[1] See docket number EPA-HQ-OPP-2015-0374 at *www.regulations.gov*
[2] See docket number EPA-HQ-OPP-2012-0181 at *www.regulations.gov*

regulatory requirements faced by the *Wolbachia*-containing mosquitoes at EPA. The distinction between the EPA review process for *Wolbachia* and that faced by Oxitec's self-limiting mosquitoes has even been noted by Nature (Waltz, 2016).

As is detailed below, horizontal gene transfer could result in *Wolbachia* effectively introducing over one thousand new genes into the recipient organism. Notwithstanding this potential for indiscriminate gene transfer, the *Wolbachia* IIT (Incompatible Insect Technique) vector control method is subjected to a substantially less rigorous regulatory review process than is a targeted genetic engineering methodology. This is directly contrary to the intent of the Coordinated Framework and to a scientifically valid risk-based regulatory process.

Such a disparate regulatory approach to two products intent on achieving similar public health ends is inconsistent with the stated Federal regulatory policy, and, as a matter of risk-based regulatory process, is without reason and justification. Oxitec's self-limiting technology and the *Wolbachia* IIT approach both have the intended purpose of end point reductions in the population of mosquitoes and involve releases of substantial numbers of non-wild type mosquitoes to the environment. Importantly, however, Oxitec's self-limiting genetic engineering is well-defined and includes only two well-studied genes, which were purposefully added and reviewed by the regulatory agencies, whereas the potential genetic modification that may result from use of *Wolbachia* pipientis, ZAP strain bacterium is wholly undefined.

Therefore, as a matter of sound regulatory action, it is incongruous for the regulatory burden placed on the *Wolbachia* IIT technology to be significantly less onerous and burdensome than the requirements imposed on Oxitec's self-limiting targeted genetic engineering technology.

As an example of the disparate regulatory treatment that Oxitec's self-limiting mosquitoes have faced, FDA's Center for Veterinary Medicine (CVM) established an Animal Biotechnology Interdisciplinary Group (ABIG) to evaluate Oxitec's technology. This ABIG included experts from FDA/CVM, CDC, and EPA. It is not clear from the record if EPA intends to consult other regulatory agencies during its consideration of the *Wolbachia pipientis Aedes albopictus* Section 3 application, but Oxitec believes that the FDA review process for Oxitec's self-limiting technology raised questions and considerations that are particularly relevant to MosquitoMate application 89668-U.

Granting a full Section 3 registration for the *Wolbachia pipientis Aedes albopictus* pesticide entails significantly greater potential risk of unreasonable adverse effects than the limited *Wolbachia* EUPs that have been granted previously. The regulatory requirements for a Section 3 registration are substantially higher than are the requirements for a limited release experimental use permit. As Oxitec has detailed in these comments, there are significant and serious risk considerations that must be addressed before EPA can determine that the proponent of registration has met the FIFRA Section 3 burden of demonstrating that the *Wolbachia pipientis Aedes albopictus* will not result in unreasonable adverse effects on the environment. In its review of the *Wolbachia pipientis Aedes albopictus* Section 3 application, EPA must, at a minimum, require the proponent of registration to adequately address these serious risk considerations. If the applicant's initial submission does not address each of these issues, EPA must require

submission of all additional data and information that is necessary to ensure that all relevant risk issues are fully addressed.

EPA's response

The *Wolbachia*, ZAP strain and Oxitec's OX513A employ different technologies. The *Aedes aegypti* OX513A Release of Insects with Dominant Lethality (RIDL) technology is unique in its approach to mosquito population suppression and relies on a novel antibiotic sensitive, transcriptional activator mechanism that is genetically engineered into *Aedes aegypti* in order to effect lethality of the insect's larvae. Sterile males are released into the environment to mate with wild females; larvae emerging from eggs laid by the females in the absence of tetracycline (as present under laboratory conditions) cannot mature into adult mosquitoes. The MosquitoMate approach to mosquito population control is based on the *Wolbachia* bacterium. *Wolbachia* is naturally present in many arthropod species, including all orders of insects and many other arthropods, where it can affect the reproductive outcome of the infected individual through cytoplasmic incompatibility. The ZAP strain of *Wolbachia* is a symbiont of the mosquito *Culex pipiens* and it is not associated with *Aedes albopictus* in the wild. When ZAP-infected male *Aedes albopictus* mosquitoes mate with ZAP-free females, their progeny do not survive, reducing the mosquito population in the subsequent generation. This self-limiting mode of action is the basis for the bacterium's insecticidal activity and reduces the likelihood of the *Wolbachia* ZAP strain to establish in wild populations.

Regarding regulatory authority over the *Wolbachia pipientis,* ZAP strain in male *Aedes albopictus* mosquitoes and the recombinant DNA (rDNA) construct engineered into the *Aedes aegypti* mosquitoes developed by Oxitec using RIDL technology, EPA clarified its jurisdiction in the May 20, 2013, response to comments regarding the experimental use permit for the release of *Aedes polynesiensis* mosquitoes infected with a strain of *Wolbachia pipientis* (EPA-HQ-OPP-2012-0181-0008; U.S. EPA, 2013). This response was later cited in the April 26, 2016 response to comments to the experimental use permit for *Wolbachia pipientis w*AlbB strain for use in *Aedes aegypti* (EPA-HQ-OPP-2015-0374-0020; U.S. EPA, 2016), where it was also discussed in the context of the Coordinated Framework for the Regulation of Biotechnology:

> EPA has jurisdiction over the *Wolbachia pipientis* bacteria that are the subject of this EUP because such bacteria constitute a substance intended for preventing, destroying or mitigating a pest, and therefore meet the definition of "pesticide" under Section 2(u) of [FIFRA]. … In contrast, because the [rDNA] construct in the genetically engineered mosquitoes developed by Oxitec using RIDL technology is being regulated by the [FDA] as a "new animal drug" under the [FFDCA], such technology does not meet the definition of "pesticide" under section 2(u) of FIFRA, which states, in part, that "… the term 'pesticide' shall not include any article that is a 'new animal drug' within the meaning of section 321(w) of Title 21…."

On January 19, 2017, FDA published Draft Guidance for Industry (GFI) #236 regarding regulation of mosquito-related products (FDA-2016-D-4482). FDA's draft guidance states: "FDA is clarifying that the phrase 'articles (other than food) intended to affect the structure or

any function of the body of man or other animals' in the FD&C Act's drug definition [21 U.S.C. 321 (g)(1)(C)] does not include articles intended to function as pesticides by preventing, destroying, repelling or mitigating mosquitoes for population control purposes." However, unless and until FDA's proposed guidance is finalized, the type or quantity of information requested by FDA in its analysis of the OX513A product as a "new animal drug" under the FFDCA remains a matter for FDA, not EPA. EPA is properly proposing to issue the present registration for the *Wolbachia* ZAP strain pesticide product pursuant to section 3(c)(5) of FIFRA (7 U.S.C. § 136a(c)(5)).

The Federal Government, in its September 16, 2016 "National Strategy for Modernizing the Regulatory System for Biotechnology Products,"[3] identified a number of actions intended to improve predictability and efficiency of regulation under the Coordinated Framework. In the National Strategy, EPA, FDA, and USDA indicated their commitment to examine their regulatory structures with the goal of clarifying how the U.S. Federal Government will regulate genetically engineered insects in an integrated and coordinated fashion to cover the full range of potential products. The agencies are working to better align their responsibilities over genetically engineered insects with their traditional oversight roles, for example, considering mechanisms that would enable EPA to regulate genetically engineered mosquitos under FIFRA when the developer claims they are intended to control population levels, and FDA to regulate them under the FFDCA when the developer makes a disease claim (see discussion of FDA's proposed Guidance for Industry #236, above). However, as long as the rDNA construct in the OX513A product is being regulated by the FDA as a "new animal drug" under the FFDCA, such technology does not meet the definition of "pesticide" under section 2(u) of FIFRA.

With regard to the comment that risk considerations must be addressed prior to registration, FIFRA section 3(c)(5) (7 U.S.C. § 136a(c)(5)) specifies four conditions that must be met prior to granting the registration of a pesticide. Two of these conditions were highlighted by one of the commenters, which are "[…] (C) [the pesticide] will perform its intended function without unreasonable adverse effects on the environment; and (D) when used in accordance with widespread and commonly recognized practice it will not generally cause unreasonable adverse effects on the environment." The term "unreasonable adverse effects on the environment" is defined in relevant part by FIFRA section 2(bb) (7 U.S.C. § 136(bb)) as "any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide…." MosquitoMate submitted the data required under 40 CFR Part 158, Subpart V that support a FIFRA section 3 registration for its *Wolbachia* ZAP strain and its use in *Aedes albopictus*. EPA has completed the risk assessments of the product and has determined that registration of this pesticide product will not result in any unreasonable adverse effects on man or the environment. With completion of the risk assessments, EPA is now making these risk assessments, along with a Proposed Decision Document, and a product label available for review and comment by the public in Docket I.D. Number EPA-HQ-OPP-2016-0205 at www.regulations.gov (see also EPA's response to comments in section III of this document, below).

[3] National Strategy for Modernizing the Regulatory System for Biotechnology Products. On September 16, 2016, the proposed Update to the Coordinated Framework for the Regulation of Biotechnology was made available by the agencies to the public. For more information, see Modernizing the Regulatory System for Biotechnology Products: Final Version of the 2017 Update to the Coordinated Framework for the Regulation of Biotechnology

In addition to ensuring that no unreasonable risk to humans or the environment is posed by the registration of a pesticide, FIFRA section 2(bb) (7 U.S.C. § 136(bb)) requires the Administrator, in considering regulatory action concerning a public health pesticide, to "[…] weigh any risks of the pesticide against the health risks such as the diseases transmitted by the vector to be controlled by the pesticide." *Aedes albopictus* mosquitoes are a known carrier of viruses that are of concern to human health, including the West Nile, chikungunya, equine encephalitis, dengue, yellow fever, and Zika viruses. Suppressing the population of *Aedes albopictus* through the release of ZAP-infected male mosquitoes has the potential to benefit human health by decreasing the chances for disease transmission, while at the same time posing minimal risk to human health and the environment. In the case of the *Wolbachia* bacterium, the majority of insects, and as a consequence the environment with which they interact, are naturally exposed to the bacterium and no negative effects have been documented as a result. *Wolbachia* are not known to infect humans. Moreover, MosquitoMate is obligated to inform EPA of any "[…] additional factual information regarding unreasonable adverse effects on the environment of the pesticide" noted during use of the pesticide product after a registration has been granted pursuant to section 6(a)(2) of FIFRA.

III. AVAILABILITY OF PRODUCT INFORMATION

Comments

From EPA-HQ-OPP-2016-0205-0010 (Nina Fedoroff) - […] As such, I recently reviewed an application to release male mosquitoes genetically modified by the company Oxitec (now a subsidiary of Intrexon) to pass a lethal gene to offspring as a mosquito control measure. The application had been submitted to the FDA, which has undertaken to regulate such mosquitoes as a "new animal drug." As part of the regulatory process, Oxitec was required to submit an Environmental Impact Assessment, which was available in its entirety for public comment, as was the application for experimental release of mosquitoes.

By contrast, application 89668-U from the MosquitoMate, Inc to register ZAP Males (*Aedes albopictus* males infected with *Wolbachia pipientis*) as a pesticide is not available for examination, although public comment on the application is requested. I therefore base my comments on examination of the relevant literature.

From EPA-HQ-OPP-2016-0205-0011 (Oxitec, Ltd.) - Based on the available record, it is not clear what level of environmental assessment has been conducted to this point. Because *Wolbachia* has been demonstrated to affect insects in the environment – changing their behaviour, disease transmission status, gene expression and biology (Marshall, 2007; Werren, *et al.*, 2008; Endersby and Hoffmann, 2013) - Oxitec believes that approval of a Section 3 registration, with the subsequent likelihood of widespread release of the modified organism, poses significant ecological risks.

If, after its initial review, EPA determines to grant the *Wolbachia pipientis Aedes albopictus* Section 3 registration, then, as part of its FIFRA public transparency policy, EPA must make available a full record of the data and information considered in the context of application

89668-U, and must provide a full and comprehensive explanation of the Agency's assessment of the significant concerns raised in these comments.

EPA's response

In accordance with section 3(c)(4) of FIFRA (7 U.S.C. § 136a(c)(4)) and 40 CFR §152.102, EPA published a Notice of Receipt (NOR) in the *Federal Register* of April 28, 2016 for the registration application of the new active ingredient *Wolbachia pipientis*, ZAP strain in *Aedes albopictus* (EPA-HQ-OPP-2016-0205-0001; 81 FR 25401). As is typical for NORs published for FIFRA section 3 applications, the information made available to the public was not extensive because EPA's review of the materials submitted by MosquitoMate was still in progress.

At this time, EPA has completed the risk assessments and the proposed decision document. In accordance with Agency policy for applications for registration of new active ingredients, certain new uses, and actions deemed by EPA to be of significant interest to the public,[4] EPA is making available the full risk assessments (which includes the review of the product characterization, manufacturing process, human health data, ecological, and efficacy data, and a modeling analyses for population dynamics), the proposed decision document, and the proposed label, while providing a comment period of 15 days[5] during which the public will have an opportunity to comment on these documents in Docket I.D. Number EPA-HQ-OPP-2016-0205 at www.regulations.gov.

IV. MOSQUITOES AS DISEASE VECTORS

Comments

From EPA-HQ-OPP-2016-0205-0002 (Anonymous) - These mosquitos have not been tested to be disease free of Zika or other viruses and until the EPA can determine that these mosquitos are safe, I opposed this application. […]

From EPA-HQ-OPP-2016-0205-0010 (Nina Fedoroff) - […] Essential as well are data on the ability of the *Wolbachia*-infected population to acquire and transmit relevant diseases, including dengue, West Nile Virus, and particularly the Zika virus.

[4] EPA considers *Wolbachia pipientis*, ZAP strain to be a new active ingredient because it is not in any currently registered pesticide products. It, therefore, is subject to the EPA's "Public Participation Process for Registration Actions" policy. See https://www.epa.gov/pesticide-registration/public-participation-process-registration-actions.

[5] For biopesticide active ingredients, such as *Wolbachia pipientis* ZAP strain, the "Public Participation Process for Registration" policy contemplates that the comment period may be less than 30 days or conducted concurrently with registration.

From EPA-HQ-OPP-2016-0205-0011 (Oxitec, Ltd.) - *Aedes albopictus* mosquitoes can naturally be superinfected with two *Wolbachia* strains (*w*AlbA, *w*AlbB), yet are still able to transmit chikungunya and dengue virus (Sinkins *et al.*, 1995; Zhou *et al.*, 1998).

Aedes aegypti artificially modified with *Wolbachia* show a reduction in dengue virus replication but virus is still found in the saliva of these engineered mosquitoes which therefore have the capacity, even if reduced, to transmit disease (Ye *et al.*, 2015).

Given that interactions between specific *Wolbachia* strains and mosquito species combinations are variable, and that *Wolbachia* has been shown to increase the vectorial capacity of some diseases in a given mosquito species, a full analysis of the capacity should be checked for all diseases

Research has shown *Wolbachia* enhances West Nile virus infection in the mosquito *Culex tarsalis*. This introduces the possibility that the *Wolbachia* infection could spread to *Culex* populations in areas where West Nile virus is a concern (Dodson *et al.*, 2014).

Research has shown *Wolbachia* can enhance malaria parasite infection in two genera of mosquitoes (Hughes *et al.*, 2012; Hughes *et al.*, 2014a; Zélé *et al.*, 2014).

Temperature impacts *Wolbachia*-malaria interaction in mosquitoes suggesting impact of transfection might vary across diverse environments (Murdock *et al.*, 2014).

Moreover, it is not unreasonable to surmise that the pathogen may evolve in response to *Wolbachia* infection in mosquitoes, with potential adverse results. Oxitec believes that additional information and analysis is required regarding interactions of host insect, the pathogen, and *Wolbachia*, to ensure that *Wolbachia* does not ultimately select for a more dangerous pathogen (Brelsfoard and Dobson, 2009).

EPA's response

With regard to questions on the potential for ZAP-infected *Aedes albopictus* to be vectors of diseases, it is correct that wild *Aedes albopictus* can carry and transmit certain viruses that are of concern to human health. The most common approach to combat mosquito-borne diseases is to reduce the number of disease vectors, e.g., through the use of larvicides and adulticides. The release of male ZAP-infected mosquitoes serves a function analogous to these classes of pesticides by reducing the mosquito population, albeit through a species-specific mode of action. At the same time, data submitted by the registrant demonstrate that the ZAP-infected individuals are not carriers of these viruses. Furthermore, male mosquitoes do not consume human or animal blood and therefore do not have a plausible probability of obtaining such viruses for subsequent transmission.

ZAP-infected *Aedes albopictus* are lab-reared in MosquitoMate's facilities, which minimizes their exposure to viruses that are of concern to human health. The individuals reared in the facility are several generations removed from the original parent lines, which were collected

from wild populations at a time when the Zika virus had not reached the western hemisphere, and in an area where the Zika virus is currently not present. As part of the EUP requirements (89668-EUP-1), MosquitoMate submitted an annual report to EPA in 2015 in which the company addressed the concern that the released ZAP-infected *Aedes albopictus* individuals could themselves be carriers of human disease-causing viruses. The company tested a total of 248 mosquitoes in its rearing facility for the presence of three pathogenic arboviruses: dengue, eastern equine St. Louis encephalitis, and West Nile. None of the 248 mosquitoes tested positive for the presence of these viruses (*see* EPA Memorandum of the Review of Product Characterization, Manufacturing Process, and Human Health Data for the Section 3 Registration of the ZAP strain *Wolbachia pipientis* in *Ae. albopictus*, September 26, 2016).

Several comments were made that the presence of the *Wolbachia* ZAP strain might increase the ability of *Aedes albopictus* to vector human diseases, with the implication that they could potentially pose a higher risk to human health than the naturally-occurring *Wolbachia* strains in these mosquitoes. The risk of a mosquito to transmit any virus, irrespective of its vectorial capacity, is limited by the acquisition and physical transmission of the virus from the infected mosquito to humans. In the mosquito family, this can only occur through females, as males do not feed on human blood and do not bite. Only lab-reared male ZAP-infected *Aedes albopictus* will be released into the environment and, as will be discussed later in this section, the expected frequency of an accidental female release is low, i.e., 1 female per 250,000 males.

With regard to the observation that *Wolbachia* enhances West Nile virus infection in *Culex tarsalis*, it is unclear how the ZAP strain could spread from *Aedes albopictus* to *Culex* species. In fact, the ZAP strain is naturally occurring in a different *Culex* species, *Culex pipiens*, and the protocol for transmission to *Aedes albopictus* involves a multi-step process in which the bacterium is extracted from *Culex pipiens* and subsequently injected into *Aedes albopictus* eggs that were cleared of their endogenous *Wolbachia* *w*AlbA and *w*AlbB strains. The observed increase in West Nile virus titer in *w*AlbB-infected *Culex tarsalis* described in Dodson *et al.*, 2014, resulted from a somatic *Wolbachia* infection into living mosquitoes and cannot be considered the same as a stably inherited *Wolbachia* infection and transmission through natural reproduction.

In response to the comment regarding the publication by Brelsfoard and Dobson, 2009, the *Wolbachia* strain *w*Mel has been shown to be able to influence the susceptibility of the fly *Drosophila melanogaster* to RNA-type viruses (Brelsfoard and Dobson, 2009). However, there is currently no direct evidence that the same effect is elicited in mosquitoes. Furthermore, any significant changes in viral abundance in the lab-reared *Aedes albopictus* would likely be noted as part of the quality assurance protocols set in place by MosquitoMate, as discussed in section V. above and in response to comments to the experimental use permit for *Wolbachia pipientis* *w*AlbB strain for use in *Aedes aegypti* (EPA-HQ-OPP-2015-0374-0020; U.S. EPA, 2016).

With regards to the Sinkins *et al.*, publication, the article does not explore the potential for *Aedes albopictus* to transmit chikungunya or the dengue viruses. It discusses the potential for superinfection in *Drosophila simulans*, but the evidence for superinfection in *Aedes albopictus* is equivocal at best, with complications in the interpretation of Restriction Fragment Length

Polymorphisms (RFLPs) in the digestion of the 16S RNA operon; for example, the *E.coli* bacterium contains several polymorphisms in 16S RNA forms even within a single isolate or strain (Suardana, 2014).

V. ENVIRONMENTAL EFFECTS

Comments

From EPA-HQ-OPP-2016-0205-0004 (Anonymous) - [...] Although there is a risk of unintended impacts to species that feed on mosquitoes through a population reduction of the food source, I believe that with appropriate and already necessary monitoring this risk will be minimal. Other mosquito species that do not transmit the Zika Virus to people will in a relatively short order fill the vacancy in the mosquito population created through this mitigation strategy, and thus any impact to the feeding species should be transient as well as minimal.

From EPA-HQ-OPP-0205-0008 (Anonymous) - [...] The larvae are a significant food source for fish. If the fish population takes a hit, that travels up the food chain and you have top level predators starved and expanding their hunting range. [...]

From EPA-HQ-OPP-2016-0205-0010 (Nina Fedoroff) - [...] The use of *Wolbachia*-infected mosquitoes to control mosquito populations is in its experimental infancy. The basic observation is that a *Wolbachia* infection spreads and takes over an insect population by a variety of mechanisms, but particularly by cytoplasmic incompatibility, such that eggs produced in an incompatible cross fail to hatch. Suppression of a local insect population is based on the release of *Wolbachia* infected males only. However, the females are not genetically sterile, hence sorting is most likely done by size and visual inspection, neither of which is 100% effective.

The possibility remains that the introduced strain will simply replace the native strain through amplification of the offspring of matings between *Wolbachia*-infected males and females, however infrequent. Suppression will likely be short-term and simple takeover of the population by *Wolbachia*-infected *A. albopictus* will be the long-term outcome.

Yet it appears that the EPA is about to approve unrestricted release of large numbers of infected insects without either an EIA, studies on its safety, or a back-up plan should the population be fully converted to the released *Wolbachia*-infected type or should other unexpected adverse effects surface.

Hence both an environmental impact assessment that addresses the probability of population replacement based on actual data is essential, a concern arising from the results of recent studies (Maurizio *et al.*, *sic*, Calvitti *et al.*, 2015).

From EPA-HQ-OPP-2016-0205-0011 (Oxitec, Ltd.) - Given that the release of females carrying the *Wolbachia* strain in the cytoplasm is to be avoided, sex sorting should approach 100%. Yet, studies have shown that mechanical sex sorting with IIT is only 90% effective in removing females. Thus, the reported inefficiency of sex sorting of *Aedes* mosquitoes infected with

Wolbachia presents a risk that must be evaluated (O'Connor *et al.*, 2012). This is particularly true in the context of a FIFRA Section 3 registration, which may result in potentially unlimited numbers of modified mosquitoes may be released.

Research has shown the mosquito's microbiome can impede vertical transmission of *Wolbachia* (Hughes *et al.*, 2014b).

The release of any females as a result of mis-sorting for any reason would allow the *Wolbachia* strain to invade the wild populations of mosquitoes in the release area, thus rendering this control approach ineffective and with the potential to spread a new strain of *Wolbachia* into the environment with unknown outcomes and consequences.

Further work is needed to define the underlying molecular mechanisms of *Wolbachia* induced reproductive modifications, particularly cytoplasmic incompatibility (Brelsfoard and Dobson, 2009).

What is the likelihood that the *Wolbachia* mosquitoes will survive and disperse once released into the environment?

What are the potential impacts of the *Wolbachia* mosquitoes in the environment, including on humans?

What are the likely consequences for the surrounding environment, should the *Wolbachia* mosquitoes survive and establish in the environment?

Any unexpected effects of *Wolbachia* could persist in the wild if any females are released, with little possibility of recall (Alphey, 2009).

In addition, for EPA to conduct an adequate scientific review of the *Wolbachia pipientis Aedes albopictus* pesticide application, EPA must address the following ecological risk assessment considerations raised in the public literature. In these comments, we highlight specific questions, risk considerations, and scientific data and information relevant to the ecological and human health risk assessment of release of *Wolbachia* mosquitoes that must be addressed prior to EPA determining that the proponent of registration of *Wolbachia pipientis Aedes albopictus* has met their burden to demonstrate that use of the pesticide will not result in unreasonable adverse effects to the environment.

Wolbachia is a bacterium residing within the cells of insects, and is passed through vertical transmission from mother to offspring. Even a single *Wolbachia* infected female could lay hundreds of eggs that would invade the wild population, thereby potentially rendering the Incompatible Insect Technique ineffective and spreading a new strain of *Wolbachia* into the environment. Modelling has shown that conditions of lower competition can favour infected females (Endersby and Hoffmann, 2013; Hancock. *et al.*, 2011; Jansen *et al.*, 2008). In other words, as a mosquito population is reduced, or if a population is already low, the chances of *Wolbachia* invading the wild population are increased.

What is the likelihood that *Wolbachia* mosquitoes can reproduce and establish in the environment into which they are released?

Oxitec believes that approval of a Section 3 registration, with the subsequent likelihood of widespread release of the modified organism, poses significant ecological risks. Therefore, to meet its FIFRA obligations, EPA must conduct a comprehensive ecological risk assessment to ensure that such a registration would not entail unreasonable adverse effects.

EPA's response

The subject of the current application for registration received a substantial number of comments that are in some cases identical to those previously addressed in response to the Notice of Receipt of the application for extension and amendment of an Experimental Use Permit (EUP) for a different strain of *Wolbachia* - the *Wolbachia pipientis* *w*AlbB strain in the mosquito *Aedes aegypti* (EPA-HQ-OPP-2015-0374-0020; U.S. EPA, 2016). Given the substantial overlap in the environmental considerations of these two microbial pesticides, such as their mode of action and demonstrated natural abundance in the environment, EPA's focus is on addressing new comments, those that are unique to the biology of the *Wolbachia* ZAP strain, or pertain specifically to the proposed registration under section 3 of FIFRA (as opposed to the issuance of an EUP). EPA will refer to the above-cited response to comments document whenever appropriate.

With regards to the comments that address the potential for accidental release of ZAP-infected female *Aedes albopictus*, male pupae are smaller than females. This sexual dimorphism is used to separate the males from the females by mechanical sorting, which is followed by visual verification. During the EUP, MosquitoMate monitored for the presence of accidentally released female ZAP-infected *Aedes albopictus* in New York, California, and Kentucky during the 2013 and 2015 field seasons. Of the 1,200 ZAP-infected released and recaptured individuals, none of them were female. MosquitoMate submitted data that estimate the probability for female releases, as a result of their manufacturing process, to be approximately 1 female per 250,000 males. The EPA reviewed the manufacturing process as part of the FIFRA section 3 registration decision, and found it to be acceptable (*see* EPA Memorandum of the Review of Product Characterization, Manufacturing Process, and Human Health Data for the Section 3 Registration of the ZAP strain *Wolbachia pipientis* in *Ae. albopictus*, September 26, 2016; EPA-HQ-OPP-2016-0205-0013). Furthermore, under FIFRA section (6)(a)(2), a pesticide registrant shall also submit to the Administrator "additional factual information regarding unreasonable adverse effects on the environment of the pesticide [...]," which would include reports of accidental releases of ZAP-infected female *Aedes albopictus*.

As stated in the response to comments to the issuance of the experimental use permit of *w*AlbB in *Aedes aegypti* (EPA-HQ-OPP-2015-0374-0020; U.S. EPA, 2016), in the case of an accidental ZAP-infected female release, females could bite a person, but this is not expected to have negative consequences because mosquitoes that are naturally infected with *Wolbachia* bite humans and no negative effects have been reported. Additionally, no antibodies are detected in relation to *Wolbachia* when *Wolbachia*-infected mosquitoes bite humans; *Wolbachia pipientis* has no known history of infecting mammals or other vertebrates (Werren *et al.*, 2008).

EPA does not expect that the reported rate of accidental female releases will result in the establishment of the ZAP strain in the wild *Aedes albopictus* population. The basis of the bacterium's pesticidal activity is that it causes bidirectional cytoplasmic incompatibility, which means that neither ZAP-infected males, nor accidentally released ZAP-infected females, can successfully reproduce with wild *Aedes albopictus*, as those individuals naturally lack the presence of this specific *Wolbachia* strain. While results achieved by the Eliminate Dengue Program [6] have shown that a new *Wolbachia* strain can successfully establish in wild *Aedes aegypti* mosquito populations, this outcome was dependent on substantial, repeated releases of high numbers of both *Wolbachia*-infected males and females, and even under these conditions not all attempts were successful (EPA-HQ-OPP-2015-0374-0020; U.S. EPA, 2016). In the case of the *Wolbachia* ZAP strain, only males are being released, and its establishment in the environment is dependent on both the accidental release of ZAP-infected females and successful mating between ZAP-infected males and ZAP-infected females. The calculated accidental release rate of 1 female per 250,000 males, and the fact that no ZAP-infected females were re-captured during the 2013 and 2015 EUPs, provide confidence that the *Wolbachia* ZAP strain will not establish in wild *Aedes albopictus* populations. Furthermore, data submitted by MosquitoMate as part of the experimental use permit also suggest that there is a reproductive cost associated with carrying the ZAP strain, resulting in lower reproductive success of ZAP-carrying versus ZAP-free individuals (*see* EPA Memorandum of the Review of Product Characterization, Manufacturing Process, and Human Health Data for the Section 3 Registration of the ZAP strain *Wolbachia pipientis* in *Ae. albopictus*, September 26, 2016; EPA-HQ-OPP-2016-0205-0013). Taken together, the probability of the *Wolbachia* ZAP strain establishing in wild *Aedes albopictus* populations is expected to be low.

As noted in the response to comments for *Wolbachia w*AlbB strain (EPA-HQ-OPP-2015-0374-0020; U.S. EPA, 2016), the comment referring to an "EIA" appears to intend to refer to either an "Environmental Assessment" (EA) or an "Environmental Impact Statement" (EIS) under the National Environmental Policy Act (NEPA). EPA is not required to perform a NEPA analysis when issuing registrations under FIFRA. *Merrell v. Thomas*, 807 F.2d 776 (9th Cir., 1986). However, EPA does conduct an environmental risk assessment for pesticide actions under FIFRA, which the Court in *Merrell* found to be equivalent to an analysis under NEPA. In the present case, because the *Wolbachia* ZAP strain is a new microbial active ingredient, MosquitoMate submitted data fulfilling the requirements under 40 CFR § 158.2150 for determining the potential risks for nontarget organisms, and EPA conducted a comprehensive ecological risk assessment for the *Wolbachia* ZAP strain and its use in male *Aedes albopictus* mosquitoes, including a risk assessment for nontarget organisms and an "effects" determination as to threatened and endangered species and their designated critical habitats. The assessments are now available for public comment (*see* EPA Memorandum of the Ecological Risk Assessment for the Section 3 registration of the microbial pesticide end-use product ZAP mosquito larvae, August 30, 2016; EPA-HQ-OPP-2016-0205).

With regards to the comment on the potential widespread release of ZAP-infected *Aedes albopictus* under a FIFRA section 3 registration (as opposed to an EUP), again, EPA conducted a

[6] See *http://www.eliminatedengue.com/program* for more information on these mosquitoes.

comprehensive ecological risk assessment for the *Wolbachia* ZAP strain and its use in male *Aedes albopictus* mosquitoes, including a risk assessment for nontarget organisms and an "effects" determination as to threatened and endangered species and their designated critical habitats (*see* EPA Memorandum of the Ecological Risk Assessment for the Section 3 registration of the microbial pesticide end-use product ZAP mosquito larvae, August 30, 2016; EPA-HQ-OPP-2016-0205). Under a FIFRA section 3 registration, although there would be no acreage limitation associated with the use of the product and therefore a greater exposure to the ZAP mosquitoes might be expected than under an EUP, EPA has determined that this pesticide product is not expected to pose a hazard to human health or the environment, and thus an increase in exposure is not expected to lead to a higher risk to human health and the environment.

As summarized in the August 30, 2016 response to comments to the experimental use permit for the *w*AlbB strain of *Wolbachia pipientis* and its use in *Aedes aegypti* (EPA-HQ-OPP-2015-0374-0020; U.S. EPA, 2016):

> …EPA anticipates beneficial impact of *Wolbachia*-infected mosquitoes on nontarget vertebrate organisms, including humans, as these pesticides are intended for suppression of mosquito vectors of viral diseases that threaten public health. Adverse impacts are not expected for humans or other vertebrates because strains of *Wolbachia pipientis* are known to establish endosymbiotic relationships only with invertebrates. Because these strains of *Wolbachia pipientis* are naturally occurring, there is already a significant history of exposure to these microorganisms, and information from the open peer-reviewed scientific literature indicates that *Wolbachia* establishes endosymbioses (some mutualistic) with approximately 60% of the insect species, as well as other invertebrates.
>
> EPA anticipates minimal or transient impact on nontarget invertebrate organisms, including nontarget mosquitoes, some of which may be beneficial, owing to the complex endosymbioses and the commonality of this bacterium in invertebrate populations. Adverse impacts are expected to the majority of targeted mosquito populations of *Aedes aegypti*, if naturally occurring females of these species successfully breed with male mosquitoes that have been infected with *Wolbachia pipientis* Strain *w*AlbB.

Some commenters raised concerns that the release of the ZAP Males and subsequent reduction in *Aedes albopictus* populations could negatively impact natural predators, such as certain species of fish and birds, by reducing their food source. The ZAP Males are a microbial pesticide that is intended to reduce *Aedes albopictus* populations, a species of mosquitoes that is invasive and non-native to the United States. Its presence was first reported in the mid-1980s and it has since commenced to spread throughout most of the United States (Moore and Mitchell, 1997; Center for Disease Control and Prevention (CDC), 2016). In contrast to other pesticide products often used to control or suppress mosquito populations, the ZAP Males are only effective against a single mosquito species, *Aedes albopictus*, which means that other mosquito populations that might also serve as a food source for predators of mosquitoes, are not affected. Much like conventional mosquitocides, including aerial sprays and larvicides, the effect of the *Wolbachia*

ZAP strain is temporary and dependent on the continual release in a designated area. It is important to note that there is no indication that the *Wolbachia* ZAP strain itself is toxic to birds or fish (*see* EPA Memorandum of the Ecological Risk Assessment for the Section 3 registration of the microbial pesticide end-use product ZAP mosquito larvae, August 30, 2016; EPA-HQ-OPP-2016-0205). At this time, EPA is not aware of any predators whose caloric intake relies significantly on *Aedes albopictus* alone. Generally, mosquitoes offer little in the way of protein, and many generalist insect predators such as bats prefer larger prey species such as lepidopterans and coleopterans (Anthony and Kunz 1977; Agosta, 2002; Jones *et al.*, 2009; Fang, 2010).

VI. EFFECTS OF ZAP-STRAIN ON *AEDES ALBOPICTUS*

Comments

From EPA-HQ-OPP-2016-0205-0011 (Oxitec, Ltd.) - Since *Wolbachia* is an endosymbiont living in the cytoplasm of the cell that has been shown to transfer genes into the nuclear genome of host insects, there is the potential that over one thousand bacterial genes could be transferred into the mosquito's nuclear genome through random integration events with unknown consequences.

The whole genome of *Wolbachia* can transfer to a host genome, meaning a host mosquito could be transformed with over one thousand new genes with unpredictable results (Kondo *et al.*, 2002; Fenn *et al.,* 2006; Dunning Hotopp *et al.*, 2007; Hou *et al.,* 2014).

Aedes albopictus is not naturally infected with *Wolbachia* ZAP strain, therefore this is an artificial infection in this species. However, *Aedes albopictus* mosquitoes can be naturally infected with certain *Wolbachia* strains (*w*AlbA, *w*AlbB), and even "super-infected" with both of these strains simultaneously. This is concerning as:

First, these naturally infected *Aedes albopictus* with *w*AlbA and *w*AlbB *Wolbachia* strains are still able to transmit chikungunya and dengue virus despite the presence of *Wolbachia* (Sinkins *et al.*, 1995; Zhou *et al.*, 1998).

Second, the consequences of any inadvertent release of female *Aedes albopictus* mosquitoes infected with the ZAP strain of *Wolbachia* mating with the naturally *w*AlbA/*w*AlbB infected *Aedes albopictus* is entirely unknown. What impact would this have on their offspring? Indeed, are there other natural *Wolbachia* strains in wild *Aedes albopictus* mosquitoes, and are all the possible interactions known? Has this been explored and addressed? Also, are there available data and information and analysis related to potential adverse effects on the environment?

It has already been shown that horizontal gene transfer (HGT) can transfer genes between *Wolbachia* and its host, in certain mosquito genera including *Aedes* (Klasson *et al.*, 2009; Woolfit *et al.*, 2009). Therefore, *Wolbachia* can genetically transform its host with functional genes with currently unknown consequences.

Widespread recombination occurs throughout the *Wolbachia* genome (Baldo *et al.*, 2006), increasing the likelihood of genes changing as the *Wolbachia* evolves. In addition *Wolbachia* has been shown to change its phenotypic effects on the host insect as it evolves (Carrington *et al.*, 2010). This could potentially change how *Wolbachia* responds to a number of factors, including how it influences host immune response and vectorial status. Therefore, potentially the vectorial capacity of *Aedes albopictus* infected with *Wolbachia* could change over time and should be continually assessed.

Importantly, however, Oxitec's self-limiting genetic engineering is well-defined and includes only two well-studied genes, which were purposefully added and reviewed by the regulatory agencies, whereas the potential genetic modification that may result from use of *Wolbachia* pipientis, ZAP strain bacterium is wholly undefined. Several studies have shown that horizontal gene transfer between *Wolbachia* and their insect hosts may result in gene transfers ranging from nearly the entire *Wolbachia* genome (>1 megabase) to short insertions (<500 base pairs) into various hosts (Kondo *et al.* 2002; Fenn *et al.,* 2006; Dunning Hotopp *et al.*, 2007; Hou *et al.,* 2014). Effectively the *Wolbachia* IIT approach could introduce over one thousand new genes into the target mosquito with unknown consequences, and if they provide a positive selection to the mosquito in the environment this could result in novel strains pervading and spreading through the population.

Introduction of *Wolbachia* into a mosquito provides the possibility to introduce over time over one thousand new genes, yet *Wolbachia* mosquitoes have not been subjected to the rigorous regulatory scrutiny that appears to be the norm for recombinant genetic modification (notwithstanding that typical genetic modification is a 'rifle shot' approach, involving very few, and fully characterised, introduced genes). As noted above, this seems entirely incongruent with the stated policy of the U.S. government as set forth in the Coordinated Framework. EPA must address the possibility of the introduction of genes of unknown function into the *Aedes albopictus* genome and the potential ecological effects that may result from such transfer.

Moreover, the possible persistence of *Wolbachia* mosquitoes themselves is a significant concern. For the reasons set forth below, each new strain of mosquito, or indeed any artificially *Wolbachia* infected insect needs to be treated as a new strain and thoroughly tested in the laboratory before any field releases.

Horizontal transmission between unrelated host species is a proven phenomenon in *Wolbachia* (Werren *et al.*, 2008). Studies have demonstrated that genetic sequences, ranging in size from single genes to entire bacterial genomes, have been transferred from *Wolbachia* to many of their insect hosts including *Aedes mosquitoes* (Kondo *et al.* 2002; Fenn *et al.,* 2006; Dunning Hotopp, *et al.*, 2007; Klasson *et al.*, 2009; Woolfit *et al.*, 2009; Hou *et al.,* 2014), and its effect on disease transmission is variable and potentially dangerous.

There is evidence that male age and overcrowding during development (i.e., under mass rearing conditions required to produce enough males for IIT to be effective) can reduce the cytoplasmic incompatibility effect in certain insects, rendering the males fertile (Yamada *et al.*, 2007) and able to spread the *Wolbachia* infection through surviving females. Thus, it is imperative that this physiological effect be investigated for *Aedes albopictus* to determine if it is a possibility in this

species. If so, EPA has to impose terms and conditions on the registration to address the potential adverse impact of this phenomenon. Specifically, as part of its Section 3 application review, EPA must require the applicant to submit data and information on age effects and rearing conditions on the cytoplasmic incompatibility penetrance in *Aedes albopictus*.

Further work is needed to define the underlying molecular mechanisms of *Wolbachia* induced reproductive modifications, particularly cytoplasmic incompatibility (Brelsfoard and Dobson, 2009).

EPA's response

Many of the issues raised above were addressed in response to the comments received on the Notice of Receipt for the extension and amendment of *Wolbachia pipientis w*AlbB strain in the mosquito *Aedes aegypti* (EPA-HQ-OPP-2015-0374-0020; U.S. EPA, 2016). EPA's focus will be on new comments and comments that address specific issues regarding the *Wolbachia* ZAP strain and its effect on *Aedes albopictus*. EPA will refer to the previous responses whenever appropriate.

Several comments were made concerning the potential for the integration of *Wolbachia* genes into the *Aedes albopictus* genome, which might subsequently genetically alter the existing wild *Aedes albopictus* populations. Gene and genome integrations occur on a multi-generational or evolutionary timescale. Whether an acquired gene would be retained and affect the organism's phenotype is furthermore a function of selection pressure. Therefore, a prerequisite for the persistence of new genetic combinations arising from the release of ZAP-infected *Aedes albopictus* in the wild is the ability to pass new genes on to the next generation. As male ZAP-infected mosquitoes are dead-end hosts, meaning that the ZAP strain will die with its host, and for other reasons discussed above in sections II., IV., and V., this is an unlikely scenario.

It is correct to suggest that potential gene integrations through horizontal or lateral gene transfer from one organism to another unrelated organism(s) can occur over evolutionary time, as is the case with all genes in all organisms. While greater than 1 million species, conservatively speaking, are infected with *Wolbachia pipientis*, common evidence for transfer, integration and expression of bacterial genes in eukaryotic organisms is a rarity, although documented in the literature in specific cases. Some of these "examples" are implied in that other competing mechanisms for significant homology amongst genes in a complex genome are pertinent, such as derivation of pseudogenes and convergent evolution.

It should be noted that lateral gene transfer from a prokaryote to a eukaryote is significantly more complex and a less likely phenomenon than prokaryotic to prokaryotic transfer and integration. This is partly due to the presence of both outer cell membranes and nuclear membranes in eukaryotes, which often preclude large, negatively charged molecules such as DNA from transiting freely. Additionally, prokaryotic genes usually lack introns and have features (e.g., promoters, ribosome binding sites) that are specific to prokaryotic expression systems, but typically fail to function in a eukaryotic genomic background. This, in large part, explains why the presence of prokaryotic genes in eukaryotes is a rare find. Absence of positive selection of

such transferred prokaryotic genes is another strong force precluding heritable transmission over time.

While *Wolbachia* can cause male killing, feminization of males, parthenogenesis, and cytoplasmic incompatibility, only the cytoplasmic incompatibility phenomenon has been associated with the ZAP strain-infected *Aedes albopictus*. As for the effects on cytoplasmic incompatibility, Calvitti *et al.* (2015) showed that *w*AlbA *Aedes albopictus* in dense rearing conditions did not decrease the cytoplasmic incompatibility effect. Islam and Dobson (2006) also showed that rearing *Aedes albopictus* with *Wolbachia* under crowded, low food conditions did not impact the effect. Yamada *et al.* (2007). cited by Oxitec, Ltd., refers to the effect in *Drosophila*, not mosquitoes. Furthermore, the data presented by MosquitoMate indicate 100% penetrance for the phenotype of a ZAP-infected male mosquito yielding cytoplasmic incompatibility after mating with a wild ZAP-free female.

VII. EXTENSION OF COMMENT PERIOD

Comment

From EPA-HQ-OPP-2016-0205-0007 (Center for Food Safety) - On behalf of the Center for Food Safety (CFS) and our 750,000 members, I am writing to request a 30-day extension of the comment period to provide input on MosquitoMate, Inc.'s application to register *Wolbachia pipientis*, ZAP Strain for use in non-biting, male *Aedes albopictus* (Asian tiger mosquito) to be released to mate with indigenous/wild female Asian tiger mosquitoes in order to control this specific species of mosquito through population suppression by prevention of egg hatch.
EPA has received an application to register a pesticide product containing an active ingredient not included in any currently registered pesticide products. Pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), EPA is required to provide notice of receipt and an opportunity to comment on this application. *Wolbachi pipientis* (ZAP Strain) will be used in male *Aedes albopictus* mosquitoes and released to mate with indigenous female mosquitoes, making this a unique pesticide.
CFS does not believe that it has sufficient time to meaningfully review, analyze, and provide comments on a potential action of this magnitude. An extension to this unique field trial encompasses an array of complicated issues and requires careful review by the public, scientists, and other experts.
Therefore, CFS urges EPA to extend the comment period by at least 30 days.

EPA's response

As discussed in EPA's response to comments in section III of this document, above, in accordance with section 3(c)(4) of FIFRA (7 U.S.C. § 136a(c)(4)) and 40 CFR §152.102, EPA published a Notice of Receipt (NOR) in the *Federal Register* of April 28, 2016 for the registration application for the new active ingredient *Wolbachia pipientis*, ZAP strain in *Aedes albopictus* (EPA-HQ-OPP-2016-0205-0001; 81 FR 25401). As is typical for NORs published for FIFRA section 3 applications, the information made available to the public was not extensive because EPA's review of the materials submitted by MosquitoMate was still in progress. To

expand the openness of the pesticide registration process, as a matter of policy EPA now provides the public "a meaningful opportunity . . . to comment on major registration decisions [e.g., new active ingredients such as *Wolbachia pipientis*, ZAP strain] at a point in the registration process when comprehensive information and analysis are available" (*see* https://www.epa.gov/pesticide-registration/public-participation-process-registration-actions). EPA decided not to extend the comment period on the NOR because it believes that those individuals wanting to comment would find the documents available through the "Public Participation Process for Registration Actions" policy to be more informative than the limited summary provided in the Notice of Receipt. As a result, comments submitted via the public participation process policy may also be more constructive and valuable than comments on the NOR in informing EPA as it moves forward toward making a decision after the public participation process policy comment period ends. As the risk assessments for the *Wolbachia* pipientis, ZAP strain application are complete, those documents, along with a proposed decision and proposed label, are provided for comment for 15 days (Docket I.D. Number EPA-HQ-OPP-2016-0205 in www.regulations.gov) in accordance with the policy described above and in EPA's response to comments in section III of this document, above.

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